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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,192,754**, on December 12, 1996, by **INSTITUT DE RECHERCHES CLINIQUES DE MONTREAL (I.R.C.M.)**, assignee of Jacques Drouin and Alexandre Philips, for "Nur-Re a Response Element Which Birds Dimers of Nur Nuclear Receptors and Method of Use Therefor".

PRIORITY DOCUMENT

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Agent certificateur/Certifying Officer

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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Nur-RE a Response Element Which Birds Dimers of Nur Nuclear Receptors and Method of Use Therefor

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ABSTRACT OF THE DISCLOSURE

The present invention relates to intracellular receptors, and methods for the modulation of transcription using same. More particularly, the invention relates to the Nur family of nuclear receptors. In general aspects, the present invention relates to the identification of a physiologically relevant response element (RE) for Nur family members, an ER-10 element, as well as to the demonstration that dimers specifically interact with the ER-10 to modulate transcription at physiologically relevant sites. The invention further relates to methods for modulating processes mediated by such nuclear receptors. In addition, the invention relates to oligonucleotide sequences that bind regulatory proteins that affect transcription, such as the Nur family of nuclear receptors, to DNA constructs comprising the oligonucleotide sequences, cells transfected with the DNA constructs, to methods of using same to provide for the controlled expression of heterologous genes, and for the the detection and recovery of new regulatory proteins. The present invention further provides bioassays for the identification of compounds as potential agonists or antagonists of transcription by the Nur family of nuclear receptors. Moreover, the invention relates to the dissection of protein-protein interactions or ligand-protein interactions involved in the modulation of transcription by the Nur family of nuclear receptors.

TITLE OF THE INVENTION

NUR-RE A RESPONSE ELEMENT WHICH BINDS DIMERS OF NUR NUCLEAR RECEPTORS AND METHOD OF USE THEREFOR.

FIELD OF THE INVENTION

The present invention relates to intracellular receptors, and methods for the modulation of transcription using same. More particularly, the invention relates to the Nur family of nuclear receptors. In general aspects, the present invention relates to the identification of a physiologically relevant response element (RE) for Nur family members, an ER-10 element, as well as to the demonstration that dimers specifically interact with the ER-10 to modulate transcription at physiologically relevant sites. The invention further relates to methods for modulating processes mediated by such nuclear receptors. In addition, the invention relates to oligonucleotide sequences that bind regulatory proteins that affect transcription, such as the Nur family of nuclear receptors, to DNA constructs comprising the oligonucleotide sequences, cells transfected with the DNA constructs, to methods of using same to provide for the controlled expression of heterologous genes, and for the the detection and recovery of new regulatory proteins. The present

invention further provides bioassays for the identification of compounds as potential agonists or antagonists of transcription by the Nur family of nuclear receptors. Moreover, the invention relates to the dissection of protein-protein interactions or ligand-protein interactions involved in the modulation of transcription by the Nur family of nuclear receptors.

BACKGROUND OF THE INVENTION

The Lipophilic hormones such as steroids, retinoids, and thyroid hormones can permeate a target cell and through their interaction with nuclear receptors, modify gene expression. Cloning and characterization of such receptors has shown that the binding of hormone to its receptor triggers an allosteric change thereof which in turns enables the hormone-receptor complex to specifically interact with a DNA target and modulate transcription. It is now widely recognized that these receptors are actually part of a superfamily of structurally related nuclear receptors which interact with chemically distinct ligands to directly affect gene expression. The importance of the nuclear receptor superfamily in maintenance of homeostasis and physiology of cells and organisms is demonstrated by the high level of conservation throughout evolution of the more than 150 members already characterized.

The nuclear receptors are characterized by (1) a DNA binding domain (DBD), responsible for the targeting of receptors to their specific response elements (RE); and (2) a ligand-binding domain (LBD), which ensures the specificity, selectivity and affinity of the binding of the ligand to its receptor (For reviews see, Mangelsdorf et al., 1995, Cell 83: 835-839; and Ibid 841-850). Characterization of the RE has shown that the RE consists of a core half-site defined by a degenerate. Xn-AGGTCA which can be configured as direct repeats (DR), inverted repeats (IR), everted repeats (ER) or nonrepeats (NR) [PCT publication number WO96/21457 published July 18, 1996]. Since the nuclear receptor recognize REs which are unique, It follows that subtle differences in the sequence of the RE or their configuration have significant effects on DNA binding of the receptor (Mangelsdorf et al., 1995, Cell 83: 835-839; and Ibid 841-850). Once bound to a RE, each receptor responds to its signal through the C-terminal ligand binding domain (LBD). The LBD contains several embedded subdomains which may include a C-terminal transactivation function, a series of heptad repeats which may serve as a dimerization interface and a poorly-delineated transcriptional suppression domain. In its natural context of the LBD, transcriptional activity through the transactivation domain, requires the addition of ligand (WO 96/21457).

A significant number of nuclear receptors are termed orphan receptors (no ligand which binds thereto has been identified). Such orphans have been identified by homology to the initial members of the superfamily in every metazoan species. It remains a significant challenge to identify a function for these orphan receptors, as well as to identify ligands and/or hormones that affect the activity thereof (Mangelsdorf et al., 1995, Cell 83: 835-839; and Ibid 841-850).

Nur77 (also known as NGFI-B, N10, NAK1, and TR3), was the first member of the Nur family of the orphan receptor subfamily of nuclear receptors to be identified. Other members of the Nur family include Nurr-1 (for Nur-related member number one; also known as RNR-1, NOT and TINUR) and NOR-1 (also known as MINOR). Nur77 distinguishes itself by its ability to bind DNA as a monomer[1,2] and by its role in TCR-induced apoptosis in T cell[3-5]. Of note, Nurr-1, the β isoform of Nur77 is described as a constitutively active orphan receptor that binds as a high-affinity monomer to an AA-AGGTCA core site and thus to the synthetic NBRE sequence (WO 96/21457). Indeed this application teaches that Nurr-1 provides a well characterized example of the paradigms of binding of nuclear receptor as a monomer to a single core site.

Nur77 has been cloned repeatedly by numerous investigators either as a mitogen-inducible gene or as an immediate early gene[6-9]. Recent work has indicated that it is widely expressed, in particular throughout the brain.

Nur77 was shown to heterodimerize with RXR to confer 9-cis retinoic acid-dependent transcription[10,11] (WO 96/21457). Two common features of nonsteroid receptors that have known ligands have been identified: the ligands are small lipophilic compounds and RXR is part of the receptor complexes. Thus, orphan receptors such as Nur77 are likely candidates for ligand-dependent activation (Mangelsdorf et al., 1995, Cell 83: 841-850).

The experiments showing heterodimerization with RXR were carried out with two synthetic DNA elements: NBRE[12] (WO 96/21457) and DR-5[11]. Synthetic NBRE was initially identified as a putative target for Nur77 by genetic selection in yeast [1]. Importantly in these experiments, Nur77 was shown to activate transcription as a monomer[1,2] (Mangelsdorf et al.,1995, Cell 83: 841-850).

There thus remains a need to identify physiological targets for the binding of Nur77 and related nuclear receptors. In addition, there remains a need to dissect the protein-protein interactions and ligand interactions relating to Nur77 and related nuclear receptors at

their physiologically relevant target sites. More particularly, it remains a need to establish whether Nur family members modulate transcription as monomers and/or homodimers and/or heterodimers.

The present invention seeks to meet these and other needs.

The description found herein refers to a number of documents, the content of which is herein incorporated by reference.

SUMMARY OF THE INVENTION

This invention concerns a natural Nur77 target sequence or response element (RE) that is responsive to physiological stimuli, in conditions where the NBRE is un - or poorly - responsive. Nur-RE is a novel response element configured as an ER-10 configuration. This novel Nur-response element (Nur-RE) mediates the physiological responses of the pro-opiomelanocortin (POMC) gene to CRH (Corticotropin Releasing Hormone) and its intracellular mediator cAMP. The Nur-RE (but not the NBRE, a single core sequence element) is also responsive to TCR-induced signals in T cell hybridomas. In contrast to NBRE binding by monomers, the Nur-RE binds homodimers of Nur77 and both halves of the Nur-RE are required for activity. Thus, the present invention is in contradistinction to the prior art. In accordance therewith

Nur-RE is shown to represent the only paradigm of Nur77, signaling that is physiologically responsive in both endocrine and lymphoid systems. The instant invention is relevant not only to Nur77, but more broadly, to the Nur family of nuclear receptors which comprises at present Nurr-1 and NOR-1.

In addition, the present invention featuresmultimeric or dimeric complexes comprising Nur family members. More particularly, it features homodimeric complexes comprising Nur family members. Evene more particularly, it concerns Nur77 homodimers. The invention also features heterodimers complexes comprising different Nur family members as well as Nur family members and non-Nur nuclear receptors. The invention also features the means to identify the specific ligands of the Nur family members of orphan receptors. Also, the invention features the means to identify factors that modulate the transcriptional activity of Nur family members through their interaction at Nur-RE. Such factors include, without being limited thereto, other nuclear receptors including Nur family members, and transcriptionally regulatory proteins.

The present invention also relates to a DNA construct comprising the NurRE or derivatives thereof, as part of an oligonucleotide operably linked to a promoter, which promoter is operably linked to a heterologous gene, wherein the DNA construct is linked in such a manner

that the heterologous gene is under the transcriptional control of the oligonucleotide sequence and promoter. Also provided is a host cell transfected with such a DNA construct.

The present invention is also related to the use of the Nur-RE of the present invention and functional derivatives thereof to screen for agents that modulate gene expression of genes having a Nur-RE region or derivative thereof in their control region. Such modulators can be used as lead compounds to design or search drugs that can modulate the level of expression of genes such as POMC or can influence TCR-induced apoptosis in T cells. The Nur-RE or derivatives thereof have utility in constructing *in vitro* or *in vivo* expreimental models for studying Nur-RE dependent transcription modulation. Such experimental models make it possible to screen large collections of natural, semisynthetic, or synthetic compounds for therapeutic agents that affect Nur-RE-dependent transcription. Once identified an agent can be formulated in a pharmaceutically acceptable fashion such as described in PCT publication number WO/9629405, published September 26, 1996, and documents cited therein. The present invention further enables the identification of signalling pathways which converge at the Nur-RE or derivatives thereof.

The present invention also concerns a method for the controlled expression of a heterologous gene of interest comprising culturing the transfected host cells containing an appropriate Nur family member(s) in the presence of a compound. Preferably, the compound in this method comprises a suspected ligand and the Nur family member comprises Nur77. In another embodiment, the method comprises the use of other nuclear receptors such as RXR.

Further, the present invention concerns a method for measuring the ability of a compound to act as an agonist of gene transcription comprising (a) contacting the compound with a transfected host cell as described above under conditions in which the heterologous gene is capable of being expressed in response to the compound, and (b) comparing the level of gene expression in step (a) with the level of gene expression from the host cell in the absence of the compound. Alternatively, the present invention also concerns a method for measuring the ability of a compound to act as an antagonist of gene transcription. In both these methods, the heterologous gene may be any appropriate reporter gene such as the heterologous gene for luciferase, chloramphenicol acetyl transferase, green fluorescent protein or β-galactosidase.

The invention further concerns ways to modulate the transcription of genes having Nur-RE target sequences or derivatives thereof. In a preferred embodiment, the present invention concerns a modulation of TCR-induced apoptosis. Such means to modulate transcription include, without being limited thereto, a targeting of the oligomerization domain (oligomerization promotion or inhibition) of Nur family members and a targeting of Nur-RE. More particularly, it concerns the dimerization domain of Nur family members.

In addition, the invention relates to methods to suppress the transcriptional activity of Nur family members comprising contacting same with excess amount of an oligonucleotide comprising Nur-RE.

The instant invention further relates to a method for identifying nuclear receptor(s) that oligomerize and/or dimerize (homo or hetero dimers) with Nur family members. Such method comprises introducing into a cell at least the DBD of a Nur family member, at least a portion of a nuclear receptor which putatively interacts with the Nur-family member, a reporter construct comprising Nur-RE or functional derivatives thereof, a promoter operable in the cell and a reporter gene, wherein the Nur-RE, promoter and reporter gene are operatively linked transcriptionally; and monitoring expression of the reporter upon

expressive to the nuclear receptor. In a preferred embodiment Nur77 is used as the Nur-family member.

The invention further concerns methods for the identification of ligands wherein such method comprise the comparison of the level of reporter gene expression which cells, comprising a reporter construct wherein the reporter gene is transcriptionally linked to Nur-RE or a functional derivative thereof, a Nur-family member(s), optionally another nuclear receptor, are exposed to a test compound and selecting those compounds which activate only the relevant combinations.

Since differential interactions among nuclear receptors can either restrict, redirect or lead to an acquisition of new ligand binding phenotypes (WO 96/21457), the present invention provides a mean to dissect the type of interactions among receptors which is operated on a physiologically relevant DNA target for Nur-family members. For example, the effect of the interaction between a Nur-family member(s) and other nuclear receptor on the modulation of transcription by Nur-family member(s) with or without ligand can be evaluated. Similar dissections could be assessed at mutated Nur-RE target sites. Also, potentiation of the effect could be evaluated by modification of Nur-RE.

The Applicant was the first to identify a natural response element for a Nur-family member of the nuclear receptor superfamily.

The applicant indeed identifies a previously undisclosed RE for Nur77 (ER-10). Moreover, the Applicant herein demonstrates that contrary to what has been described in the litterature, the monomeric and heterodimeric forms of transcription transactivator assumed by Nur family members, are not the only transcriptionally active forms thereof. Indeed, the applicant herein identifies homodimers of Nur77. Moreover, the homodimeric form of Nur77 is shown to be a physiologically relevant form of Nur transcriptional complexes on the genes tested. Using NBRE as a target site, Nur77 was shown to heterodimerize with retinoid X receptor (RXR), the complex thereby becoming responsive to 9-*cis*RA. The identification of a physiological target sequence for Nur77, now enables the assessment of the validity of the Nur77-RXR interaction thereon and opens the way to the dissection of relevant interactions relating to the control of gene expression through the Nur family of nuclear receptors. Indeed, the applicant has surprisingly discovered that the Nur-RE dependent action of Nur77 is not activated by 9-*cis*RA, in the presence or absence of RXR.

While the instant invention is demonstrated by experiments performed with Nur77, the invention is not so limited. Other Nur family members as identified above, or isoforms thereof may be used using the same principle taught herein. Three Nur family members

(Nur77, Nurr-1 and NOR-1) can activate transcription from reporter genes containing the Nur-RE, but hardly from NBRE containing reporters (data nor shown). It follows that the results described herein for Nur77 are most likely applicable to the other Nur family members. Moreover, it is likely that various members of this family may act and bind either as homodimers (as shown herein for Nur77) or as heterodimers or other types of multimers on Nur-RE (ER-10) containing reporter target genes. The same applies for the use of the Nur-RE. As demonstrated hereinbelow, and as known to the skilled artisan, oligonucleotide sequences can tolerate some changes without affecting their biological activity. As evidenced below, the Nur-RE can be mutated in the M2 region without affecting its physiologically relevant interaction with Nur77. Specific mutations of Nur-RE and a comparison of their effect on transcription modulation by Nur family members (using methods of the present invention) could permit a determination of a consensus sequence necessary and sufficient for specifically binding multimers comprising at least one member of the Nur family of nuclear receptors.

As used herein, the term "physiologically relevant" is meant to describe interactions which can modulate transcription of a gene in its natural setting.

The term "oligonucleotide" or "DNA" molecule or sequence refers to a molecule comprised of the deoxyribonucleotides adenine (A), guanine (G), thymine (T) and/or cytosine (C), in a double-stranded form, and comprises or includes a "regulatory element" according to the present invention, as that term is defined herein. The term "oligonucleotide" or "DNA" can be found in linear DNA molecules or fragments, viruses, plasmids, vectors, chromosomes or synthetically derived DNA. As used herein, particular double-stranded DNA sequences may be described according to the normal convention of giving only the sequence in the 5' to 3' direction.

"Regulatory element" refers to a deoxyribonucleotide sequence comprising the whole, or a portion of, an oligonucleotide sequence to which an activated transcriptional regulatory protein, or a complex comprising one or more activated transcriptional regulatory proteins, binds so as to transcriptionally modulate the expression of an associated gene or genes, including heterologous genes.

"Transcriptional regulatory protein" refers to cytoplasmic or nuclear proteins that, when activated, bind the regulatory elements/oligonucleotide sequences of the present invention either directly, or indirectly through a complex of transcriptional regulatory proteins or other adapter proteins, to transcriptionally modulate the

activity of an associated gene or genes. Thus, transcriptional regulatory proteins can bind directly to the DNA regulatory elements of the present invention, or can bind indirectly to the regulatory elements by binding to another protein, which in turn binds to or is bound to a DNA regulatory element of the present invention. As used herein, transcriptional regulatory proteins, include, but are not limited to, those proteins referred to in the art as signal transducers and activators of transcription (STAT) proteins and nuclear receptors, as well as to all substantially homologous analogs and allelic variations thereof.

"Transcriptionally modulate the expression of an associated gene or genes" means to change the rate of transcription of such gene or genes.

"DNA construct" refers to any genetic element, including, but not limited to, plasmids, vectors, chromosomes and viruses, that incorporate the oligonucleotide sequences of the present invention. For example, the DNA construct can be a vector comprising a promoter that is operably linked to an oligonucleotide sequence of the present invention, which is in turn, operably linked to a heterologous gene, such as the gene for the luciferase reporter molecule. "Promoter" refers to a DNA regulatory region capable of binding directly or indirectly to RNA polymerase in a cell and initiating transcription of a downstream (3'

direction) coding sequence. For purposes of the present invention, the promoter is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter will be found a transcription initiation site (conveniently defined by mapping with S1 nuclease), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CCAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences. "Gene" refers to a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein. A "heterologous" region of a DNA construct (i.e. a heterologous gene) is an indentifiable segment of DNA within a larger DNA construct that is not found in association with the other genetic components of the construct in nature. Thus, when the heterologous gene encodes a mammalian gene, the gene will usually be flanked by a promoter that does not flank the structural genomic DNA in the genome of the source organism. A promoter of a DNA construct, including an oligonucleotide sequence according to the present invention, is "operably linked" to a heterologous gene when the presence of the

promoter influences transcription from the heterologous gene, including genes for reporter sequences such as luciferase, chloramphenicol acetyl transferase, β-galactosidase, and the like. Operably linked sequences may also include two segments that are transcribed onto the same RNA transcript. Thus, two sequences, such as a promoter and a "reporter sequence" are operably linked if transcription commencing in the promoter will produce an RNA transcript of the reporter sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

A host cell has been "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA has been introduced inside the cell. The transfecting DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transfecting DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transfected cell is one in which the transfecting DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA.

The oligonucleotide sequences of the present invention can also comprise multimers of two or more "units" of the basic regulatory elements. In this regard, such multimer oligonucleotide sequences can, as a practical matter, contain from about 2 to 15 units of the same or different regulatory elements according to the present invention. However, theoretically, there is no limit to the number of regulatory elements within such a multimer oligonucleotide sequence. Such multimeric oligonucleotide sequences are useful as probes for detecting, isolating and/or purifying transcriptional regulatory proteins and in particular Nur family members or factors that interact therewith. Further, when used in a DNA construct, including a promoter and heterologous gene, according to the present invention, a multimer of the regulatory elements can enhance the expression of the gene from the DNA construct in response to various molecules such as transcription factors, nuclear receptors, ligands, or compounds (i.e. antagonist).

The regulatory elements and/or oligonucleotide sequences of the present invention will also prove useful in detecting, isolating and purifying new transcriptional regulatory proteins that display binding specificity to the regulatory elements/oligonucleotide sequences of the present invention either directly or indirectly, through their interaction with factors specifically interacting with the RE/oligonucleotide

sequences. Further, it is contemplated that these regulatory elements/oligonucleotide sequences will prove particularly useful in the discovery of novel proteins which modulate transcription through a direct or indirect interaction with the RE/oligonucleotides of the invention. In this regard, detection of such novel transcriptional regulatory proteins can be accomplished with the following technique. Techniques which can be used for such identification are know in the art and includes techniques described in PCT publication number WO 95/28482 published October 26, 1995 and citations found therein. Such techniques can be in vitro methods which comprise the use of extracts of the nucleus and cytoplasm of cells, electrophoretic mobility shift assays, and in vitro translations. In addition, they can also be in vivo methods, based on the use of suitable DNA constructs in accordance with the present invention.

The regulatory elements/nucleotide sequences of the present invention can also serve as a "probe", similar to those used in a variety of nucleic acid detection systems well known in the art, except that the probes of the present invention are used to detect proteins, rather than a nucleic acid sequences, which specifically bind to the regulatory elements/oligonucleotide sequences of the present invention. DNA probes according to the present invention preferably include the regulatory elements alone, or as part of a longer oligonucleotide

sequence of the present invention, labeled with a detectable label, such as a radioisotope, an enzyme, a fluorescent label, a chemical label, or a modified base. In addition, multimers of the oligonucleotide sequence of the present invention are also contemplated as probes.

Thus, the present invention provides a method for detecting the presence of novel transcriptional regulatory proteins in a sample. Such samples are preferably biological samples, including, but not limited to, cells, cell culture supernatant, cell or tissue extracts, or particular fractions thereof, and other biological fluids such as blood, sera, urine, saliva, etc. Such regulatory proteins could also be detected from in vitro preparations thereof, such as for example in vitro translated proteins. Binding of the probe containing the regulatory elements/oligonucleotide sequences of the present invention to a transcriptional regulatory protein in the sample may be detected by any appropriate means known in the art. For example, direct or indirect, or competitive binding assays may be used. Once detected, the novel transcriptional regulatory protein can be separated and purified from the probe-protein complex by any of a variety of techniques well known to those of skill in the art.

In one embodiment, the regulatory element/oligonucleotide sequence of the present invention is immobilized on a solid support or carrier. As used herein "solid phase carrier or

support" refers to any support capable of binding the oligonucleotide sequences/DNA regulatory elements of the present invention. Methods for coupling nucleic acids to the solid phase, the solid phase substances useful in these methods, and the means for elution of the proteins from the bound ligand, are well known to those of skill in the art.

The recombinant DNA constructs in accordance with the present invention can be constructed using conventional molecular biology, microbiology, and recombinant DNA techniques well known to those of skill in the art (i.e. Sambrook et al, 1989, Molecular Cloning: A Laboratory Manual). With a suitable DNA construct transfected into a host cell, the present invention provides a method for the controlled expression of a gene of interest. Alternatively, when the DNA construct comprises a reporter sequence, such as the gene for luciferase, transfection of the DNA construct into a host cell provides a convenient means for measuring the transcriptional activity of a reporter product in response to a signaling molecule, to a suspected ligand or to the presence of transcriptional factors or transcriptional modulators.

As used herein, agonists or antagonists of gene transcription, through the RE of the present invention, include compounds that intervene at any point within the signaling pathway from interaction between the signaling molecule and a cell surface or intracellular receptor

through activation of one or more transcriptional regulatory proteins and binding of the same to DNA regulatory elements, the end result of which is modulation of gene transcription by Nur family members. Further, as used herein, agonists and antagonists of gene transcription also include potentiators of known compounds with such agonist or antagonist properties. They also include compounds that may facilitate or impair dimerization of regulatory proteins in condition where dimerization is an important or essential event for modulation of gene expression. Agonists can be detected by contacting the transfected host cell with a compound or mix of compounds and, after a fixed period of time, determining the level of gene expression (e.g. the level of luciferase produced) within the treated cells. This expression level can then be compared to the expression level of the reporter gene in the absence of the compound(s). The difference between the levels of gene expression, if any, indicated whether the compound(s) of interest agonize the activation of intracellular transcriptional regulatory proteins in an analogous fashion to a known agonist of transcription. Further, the magnitude of the level of reporter product expressed between the treated and untreated cells provides a relative indication of the strength of that compound(s) as an agonist of gene transcription via a transcriptional regulatory protein pathway. Alternatively, such a transfected host cell can be used to find antagonists

of known agonists of transcription, utilizing host cells transfected with the DNA construct according to the present invention. In such an assay, the compound or compounds of interest are contacted with the host cell in conjunction with one or more known agonists held at a fixed concentration. The extent to which the compound(s) depress the level of gene expression in the host cell below that available from the host cell in the absence of compounds, but presence of the known agonist, provides an indication and relative strength of the antagonist properties of such compound(s).

Thus, the present invention concerns methods to assay for agonists and antagonists of gene transcription utilizing the regulatory elements/oligonucleotides of the present invention in appropriate DNA constructs and transfected host cells. Further, the agonist and antagonist compounds discovered utilizing these methods can serve as pharmaceutical agents in the intervention of various disease states and conditions, or to ameliorate disease states wherein a modulation of transcription would be beneficial, a non-limiting example thereof includes TCR-induced apoptosis.

Having herein identified dimers as a physiologically relevant modulator of transcription, the present invention provides means to modulate transcription by affecting dimerization (or other types of

multimerization). For example, an inhibition of Nur77 dimerization could reverse the Nur77-dependent TCR-induced apoptosis in T cells. Alternatively, promoting dimerization could enhance this TCR-induced apoptosis. The discovery that Nur family members and glucocorticoids can interact to affect Nur-RE-dependent transcription opens the way to a modulation of therapeutic actions of glucocorticoids with the Nur-RE-dependent signalling path way.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non restrictive description of preferred embodiments thereof, given by way of example only with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In the appended drawings:

Figure 1 shows that the POMC gene promoter contains a Nur response element (Nur-RE) that confers responsiveness to Nur77 and CRH in AtT-20 cells, and to TCR activation in T cell hybridomas. A) CRH (10^{-7} M) treatment of AtT-20 cells leads to a transient induction of Nur77 mRNA as assessed by Northern blot. Cellular β-actin mRNA was measured by hybridization on the same blot as control. Total AtT-20 cell

RNA (20 µg) was used as previously described [26]. **B)** Localization of Nur-RE and comparison of its activity with that of NBRE. The rat POMC promoter (-480 to +63 bp) fused to the luciferase reporter (construct 1) was previously described [16]. The mutations of the NBRE present within the nGRE (construct 2) and of Nur-RE (construct 3) contain transversions of 15 and 10 bp, respectively. Constructs 6 and 7 contain trimers of Nur-RE and NBRE (28 bp) inserted upstream of a minimal POMC promoter (-35 bp to + 63 bp). **C)** Co-localization of CRH responsiveness with the Nur-RE. **D)** The Nur-RE confers responsiveness to both Nur77 overexpression and treatment with anti-CD3 in the T cell hybridoma D0 11.10;

Figure 2 shows a blockade of CRH and forskolin responsiveness by a dominant negative mutant of Nur77 (dNur77). The response of four reporter plasmids to CRH (10^{-7} M) and forskolin (10^{-7} M) was tested after lipofection in AtT-20 cells. the reporters were: POMC-luc, Nur-RE-luc, NBRE-luc, and RSV-luc as a negative control reporter. Lipofection was performed as described in the legend to Figure 1 and the expression plasmid for the Nur77 dominant negative mutant was used at 3 µg/dish. This dominant negative mutant was described previously[4] and shown to block TCR-induced apoptosis in T cells.; and

Figure 3 shows the characterization of Nur-RE. A) Binding of *in vitro* translated Nur77 to Nur-RE and NBRE. The position of monomeric (mono) and dimeric (dimer) complexes is indicated by arrows. Comperitor oligonucleotides were used at 100-fold molar excess. B) Binding curve of Nur77 in the presence of increasing concentrations of Nur-RE and NBRE. C) Quantitation of binding experiments shown in B. Each band was quantitated using phosphor-Imager™. D) Localization of Nur-RE by DMS interference. End-labeled coding (C) and non-coding (NC) strands of the Nur-RE were used for DMS interference of Nur77 binding. DMS methylation partially revealed A residues in addition to guanosine. Residues that interfere with binding are boxed on either sides of the gels, and they are indicated by arrowheads on the Nur-RE sequence below. Arrows between the strands indicate the position of the Nur-RE half-sites which are related to the consensus AAAGGTCA. The position of transversion mutations (M1, M2, M3) used in binding experiments shown in E is indicated below the sequence. In addition, nucleotides mutated in the linker scanning mutant used in Figure 1B are indicated by a line. E) Binding of Nur77 to the wild-type and mutant Nur-RE. The position of each mutation is indicated below the sequence in D. F) Relative activity of Nur-RE and mutants compared to that of NBRE. Lipofection was carried out as in Figure 1B. The mutant Nur-RE has 2 bp

replaced at positions -390/-391 in the POMC promoter[15]. Methods. For gel retardation experiments, Nur-RE or NBRE oligonucleotides were 3'-end-labeled using Klenow polymerase and purified on polyacrilamide gels. Binding conditions and DMS interference were as previously described[16,30]; and

Figure 4 shows the interrelationship between Nur77-dependent transcriptional modulation and that dependent on glucocorticoids. D and F demonstrate that glucocorticoids reverse the Nur-RE-dependent Nur77 transcriptional activation. Moreover, this reversion of Nur77 is also manifested phenotypically as TCR-induced apoptosis is reversed by the addition of glucocorticoids (data not shown).

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawings which are examplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Methods. Transfections in AtT-20 cells (B and C) were performed by lipofection (lipofectin, Gibco) using exponentially growing AtT-20 cells (7.5 X 10^5) in 35 mm Petri dishes. Cells were grown in DMEM with 10% fetal

calf serum stripped with dextran-coated charcoal[29]. Cells and media were harvested 16 h after lipofection. Each sample for lipofection contained a total of 1.5 µg DNA, including 300 ng reporter plasmid, 300 ng RSV-GH as internal control. 100 ng of pRSV-Nur77 expression vector, 100 ng RSV-GR, and total DNA was completed to 1.5 µg with pSP64. DO 11.10 cells were electroporated using a Biorad instruments at 250 V, 960 µFarad, and 10 µg each of reporter plasmid and expression vectors. CRH was added at 10^{-7} M and anti-CD3 (clone 145-2C11) was used at 1 µg/ml to coat dishes. The data represent the means ± SEM of 3 to 5 experiments performed each in duplicate.

As Nur77 was previously implicated in regulation of the hypothalamo-pituitary-adrenal axis [12-14], we tested whether it is induced in POMC-expressing cells in response to CRH and whether it acts on transcription on the POMC gene (Fig. 1). Nur77 expression is rapidly induced in response to CRH (Fig. 1A). Overexpression of Nur77 was found to increase transcription of a POMC luciferase reporter (Fig. 1B, construct 1) and mutagenesis of a NBRE sequence (found within a previously described nGRE [15]) did not prevent this effect (construct 2). However, deletion of the distal region of the promoter [16] was found to abolish activity (construct 4) and further mapping of the responsive sequences using a variety of deletions and linker scanning mutants (data

not shown) led to the identification of a target sequence, the Nur-RE, centered around -395 bp. A specific linker scanning mutation of the Nur-RE abolished responsiveness to Nur77 (construct 3). In order to clearly define sequences required for Nur response, a Nur-RE oligonucleotide was inserted in three copies upstream of a minimal promoter, and this response element was found to confer high responsiveness to Nur77 (construct 6). In this context, the Nur-RE is at least 40 times more responsive than the NBRE (construct 7, note scale difference).

Since the Nur orphan receptors have been implicated in signaling [3,4,12-14,17], we tested whether the stimulatory effect of CRH on POMC transcription might be mediated through this pathway. When the same promoter deletions were tested for responsiveness to CRH (Fig. 1C), it was found that the Nur-RE confers responsiveness to CRH as the linker scanning mutation of this element (Fig. 1, construct 3) abolished responsiveness to the hypothalamic hormone, and oligomerization of the response element leads to a greatly enhanced response (construct 6). In view of the importance of the Nur pathway in TCR-mediated signaling, we tested the relative activity of the Nur-RE and NBRE in T cell hybridomas following Nur77 expression and anti-CD3 activation of TCR signaling (Fig. 1D). Whereas the Nur-RE reporter was induced by TCR activation, the NBRE reporter was not. Thus, the Nur-RE provides a

paradigm for naturally occurring target sequences of the Nur orphan receptor signaling pathway.

Since it was previously suggested that CRH may mediate its effect through cAMP and PKA, the response of the Nur-RE to forskolin was also tested (Fig. 2). Interestingly, the Nur-RE reporter was also responsive to forskolin but less so than to CRH, suggesting that CRH may induce other pathways in addition to the cAMP pathway. In order to demonstrate the importance of the Nur pathway in activation of the POMC promoter in response to CRH and cAMP, we used a dominant negative mutant of Nur77 (dNurr77) that had previously been shown to block TCR-induced signals and apoptosis in T cells [4]. Overexpression of dNur77 decreased basal POMC promoter activity and completely blunted CRH-induced activity (Fig. 2). In addition, dNur77 blunted the response of the Nur-RE reporter to CRH and forskolin. The weak activity and responsiveness of the NBRE-containing reporter was also decreased by dNur77. The complete reversal of CRH-induced POMC transcription by dNur77 suggests that this pathway is solely responsible for the transcriptional actions of CRH in AtT-20 cells.

The interaction of Nur77 with Nur-RE was investigated directly in binding studies using *in vitro* translated Nur77. Surprisingly, these binding experiments indicated that the Nur-RE binds homodimers

of Nur77 in contrast to the monomeric interaction of this receptor with NBRE (Fig. 3A). The prevalence of dimeric complexes in gel retardation experiments suggests that dimer formation is co-operative (Fig. 3B and C). In competition experiments, both Nur-RE and NBRE exhibited similar specificity of binding (Fig. 3A). The interaction of Nur77 with Nur-RE was further defined using the DMS interference method (Fig. 3D). This analysis indicated that two Nur77 moieties interact with octamer motifs that are found in an inverse orientation and separated by 6 bp. Each motif is loosely related to the NBRE: AAAGGTCA (Fig. 3D). The upstream octamer motif is the most conserved by comparison to NBRE. The linker scanning mutation used to localize the Nur-RE (Fig. 1A, construct 3) was targeted to this upstream motif as indicated in Figure 3D. However, this upstream motif is insufficient on its own to confer Nur-RE activity (see below).

The importance of each motif for binding of Nur77 homodimers was confirmed in gel retardation experiments; indeed, mutation of either motif (mutants M1 and M3) prevented formation of homodimer complexes whereas mutation of intervening sequences (mutant M2) did not (Fig. 3E). The binding of Nur77 monomers to the M1 and M3 mutants is consistent with the observation that the Nur-RE half-sites are similar to NBRE. The Nur-RE is somewhat unusual in sequence

in that its two inverted half-sites or NBRE-related motifs are separated by 6 or 10 bp depending on whether one considers the octamer sequence recognized by Nur77 [2] or the hexamer motif used to classify other nuclear receptor target sites [18,19]; thus, in the usual nomenclature [19], the Nur-RE is an ER-10 element. Previous work has shown that DNA recognition by Nur77 (NGFI-B) extends by 2 bp upstream of the canonical hexanucleotide AGGTCA by comparison to other nuclear receptors and that this interaction involves amino acid residues outside of the zinc finger domain [2]. These two A residues are present in each half-site of the Nur-RE (Fig. 3D) suggesting that this mode of DNA recognition is used as it is for Nur77 monomer interaction with NBRE. Replacement of the first A by a G in one motif sufficed to abolish Nur-RE activity in response to Nur77 overexpression, as did the deletion of an octamer motif (Fig. 3F). The activity of those Nur-RE mutants is the same as that of the NBRE reporter: it is not clear whether this activity is due to the action of three Nur77 monomers or to weakly binding dimers. However, in the context of the POMC promoter, a single NBRE sequence is totally unresponsive to CRH or Nur77 overexpression while a single Nur-RE appeared sufficient for responsiveness (Fig. 1B and C).

The identification of the Nur-RE as a target for binding of Nur77 dimers raises the question of the biological relevance of the

NBRE since this target sequence was originally identified in yeast [1]. Later, NBREs were identified by homology in putative Nur77 target genes, in particular, in genes encoding adrenal steroidogenic enzymes [20], but formal proof that these sequences confer biological response other than in transfection experiments is lacking. Despite its importance in TCR-induced apoptosis [3-5], there are as yet no known downstream genes of the Nur77 pathway in T cells. The identification of a potent naturally occurring Nur-RE should facilitate the search for Nur77 target genes which lie downstream of Nur77 in the signaling cascade leading to T cell apoptosis.

The convergence of CRH and cAMP signals at the Nur-RE in the POMC gene may seem surprising. However, the POMC promoter does not contain a CRE element and forskolin did not fully mimic the effect of CRH on Nur-RE reporters (Fig. 2), suggesting that forskolin effects may be indirect. CRH was also shown to elevate intracellular Ca^{++} [21] and Ca^{++}-dependent signals have been implicated in Nur activation in T cells [22,23]. Thus, it may be that different signals converge on Nur77 to modulate POMC transcription. The POMC promoter has two potential targets for Nur77: the Nur-RE and the NBRE which is contained within the nGRE [15]. The latter binds Nur77 monomers and exhibits a similar activity as NBRE in trans-activation experiments

(data not shown). Although this putative Nur target site was not found to contribute responsiveness to CRH in AtT-20 cells (Fig. 1B, C), it may play a role under some physiological conditions or in other POMC-expressing cells. This latter possibility is not unlikely since the activity of the upstream Nur-RE is dependent on corticotroph-specific recognition of flanking promoter elements [24]. Indeed, the tissue-restricted HLH factor NeuroD/BETA2 (G. Poulin and J. Drouin, in preparation), and the *bicoid*-related factor Ptx1 which are important determinants of corticotroph-specific POMC transcription, bind just downstream of the Nur-RE [25]. In conclusion, the Nur77 signaling pathway appears to be an important positive regulator of the hypothalamo-pituitary-adrenal axis as Nur77 and related factors [26,27] mediate activation of the axis at all three levels, hypothalamus (CRH), pituitary (POMC), and adrenals (steroidogenic enzyme-coding genes)

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

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WHAT IS CLAIMED IS:

1. A double stranded oligonucleotide sequence comprising a response element, wherein said response element comprises two half site sequences configured as an everted repeat (ER) separated by 10 bp by comparison with the core hexamer motif classifying nuclear receptor target sites, wherein said half site sequences share homology with NBRE, and wherein the response element binds to nuclear receptors.

2. The oligonucleotide sequence of claim 1, wherein the response element binds a dimer of nuclear receptors.

3. The oligonucleotide sequence of claim 2, wherein the dimer is a homodimer comprised of a member of the Nur family of nuclear receptors.

4. The oligonucleotide sequence of claim 3, wherein the dimer is a heterodimer, wherein one partner of the heterodimer is a member of the Nur family of nuclear receptors.

5. A double stranded oligonucleotide sequence comprising a response element, wherein said response element comprises two half site sequences configured as an everted repeat (ER) separated by 10 bp, by comparison with the core hexamer motif classifying nuclear receptor target sites, wherein said half site sequences are selected from the group consisting of ATATCA, AAATATCA, ATGCCA, AAATGCCA, AAGGTCA, and AAAGGTCA, complements, or functional derivatives thereof, and wherein the response element binds to nuclear receptors.

6. The oligonucleotide sequence of claim 5, wherein the response element is selected from the group consisting of :

GTGATATTTXXXXXXAAATGCCAG, TGATATTTXXXXXXAAATGCCA,
GTGATATTTXXXXXXAAATATCAC, TGATATTTXXXXXXAAATATCA,
CTGGCATTTXXXXXXAAATGCCAG, TGGCATTTXXXXXXAAATGCCA,
QTGACCTTTXXXXXXAAAGGTCAQ, TGACCTTTXXXXXXAAAGGTCA,
QTGUYATTTXXXXXXAAATUYCAQ, TGUYATTTXXXXXXAAATUYCA,
GTGATATTTACCTCCAAATGCCAG, TGATATTTACCTCCAAATGCCA,
GTGATATTTACCTCCAAATATCAC, TGATATTTACCTCCAAATATCA,
CTGGCATTTACCTCCAAATGCCAG, TGGCATTTACCTCCAAATGCCA,

QTGACCTTTACCTCCAAAGGTCAQ,TGACCTTTACCTCCAAAGGTCA, QTGUYATTTACCTCCAAATUYCAQ,TGUYATTTACCTCCAAATUYCA, complements and functional derivatives thereof, wherein X is independently selected from A,T,C,or G, U is a purine, Y is a pyrimidine, and Q is C or G.

7. A DNA construct comprising the oligonucleotide sequence of claim 1 operably linked to a promoter, which promoter is operably linked to a heterologous gene, wherein the DNA construct is linked in such a manner that the gene is under the transcriptional control of the oligonucleotide sequence and promoter.

8. The DNA construct according to claim 7, wherein the oligonucleotide sequence comprises a multimer of at least one response element.

9. The DNA construct of claim 7, wherein the heterologous gene is a reporter gene.

10. A host cell transfected with the DNA of claim 7.

11. A host cell transfected with the DNA of claim 9.

12. A method for controlled expression of a heterologous gene of interest comprising culturing a host cell according to claim 10 in the presence of appropriate regulatory proteins.

13. The method according to claim 12, wherein the regulatory protein comprises a member of the Nur family of nuclear receptors.

14. A method for detecting a modulator of transcription at a Nur-RE, comprising contacting a sample with the host cell according to claim 11, and comparing the level of expression of the reporter gene in the presence of the sample and in the absence thereof.

15. A method for measuring the ability of a compound to modulate transcription at a Nur-RE comprising:

a) contacting the compound with the host cell according to claim 10 or 11, under conditions conducive to the expression of the heterologous gene in response to the compound; and

b) comparing the level of gene expression in step a) with the level of gene expression from the host cell in the absence of the compound.

16. The method of claim 15 to identify a ligand selective for Nur family transcriptional complexes.

17. A multimeric complex comprising at least one member of the Nur family of nuclear receptors.

18. The multimeric complex of claim 17, wherein said multimeric complex is a homodimer.

19. The multimeric complex of claim 17, wherein said multimeric complex is a heterodimer, wherein at least one member of said heterodimer is a member of the Nur family of nuclear receptors.

20. A multimeric complex comprising at least one member of the Nur family of nuclear receptors, wherein said complex

specifically interacts with the oligonucleotide sequence of claim 1, under physiologically relevant conditions.

21. A method for treating a host suffering from a disease or condition characterized by an involvement therein of a gene being transcribed in a Nur-RE-dependent fashion, comprising the step of administrating to said host a composition comprising an effective amount of a compound which affects multimerization of a complex comprising at least one member of the Nur family of nuclear receptors and/or which affects interaction of a member of a Nur family of nuclear receptors with said Nur-RE.

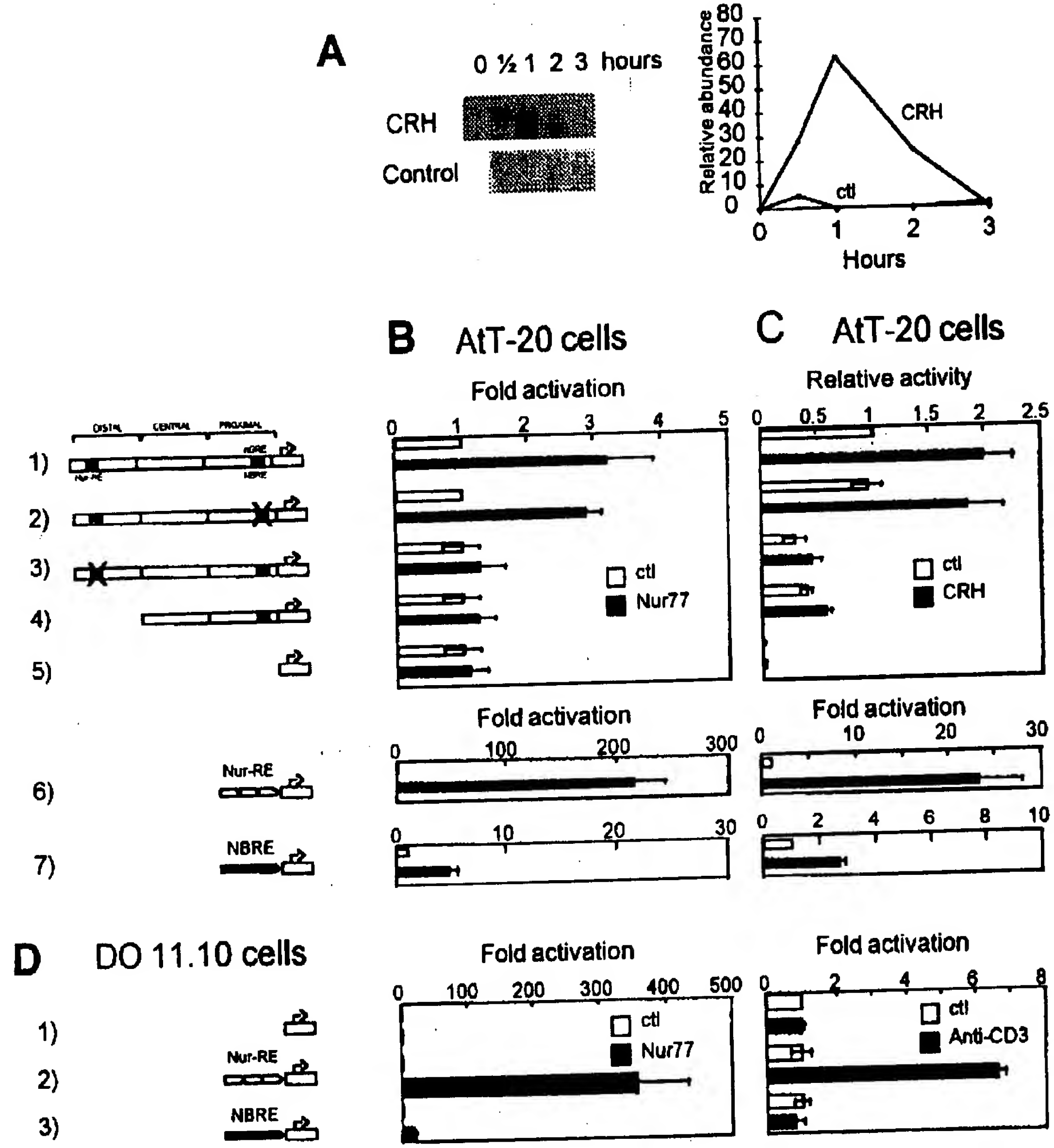

FIG. 1

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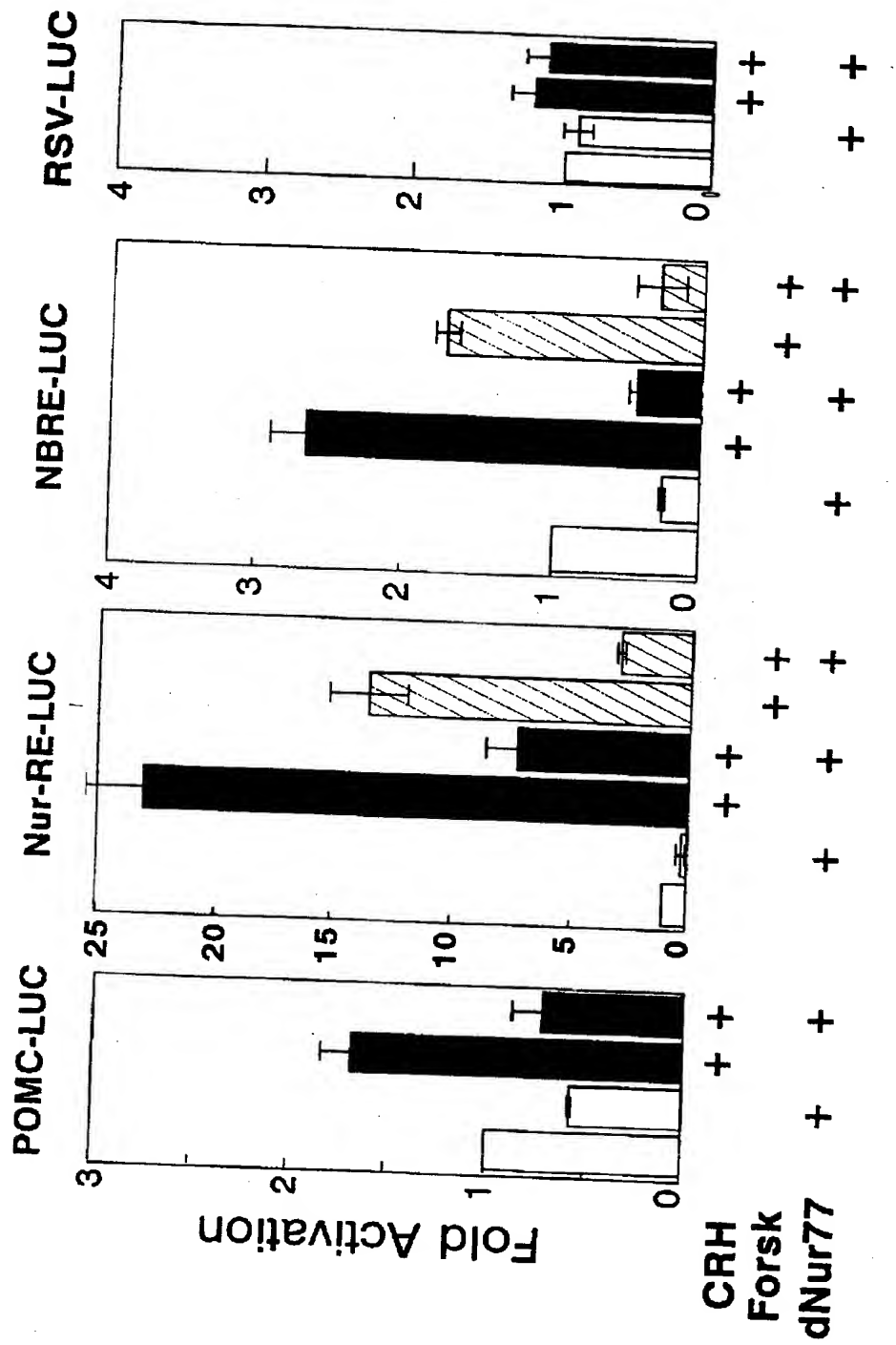
FIG. 2



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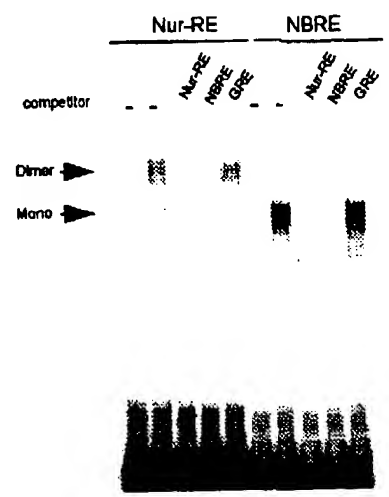

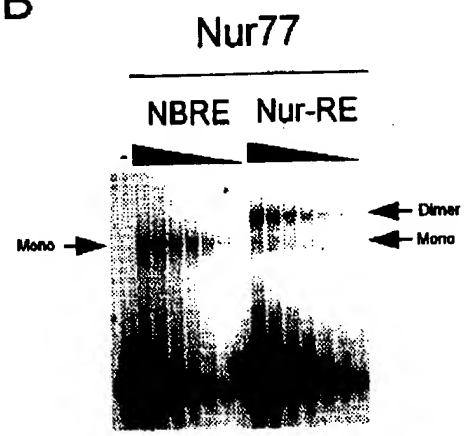

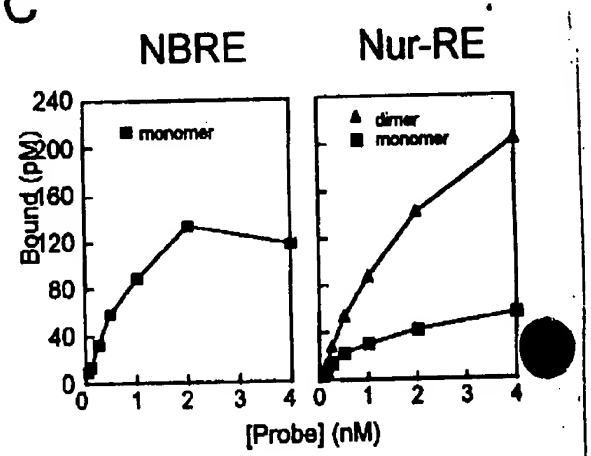

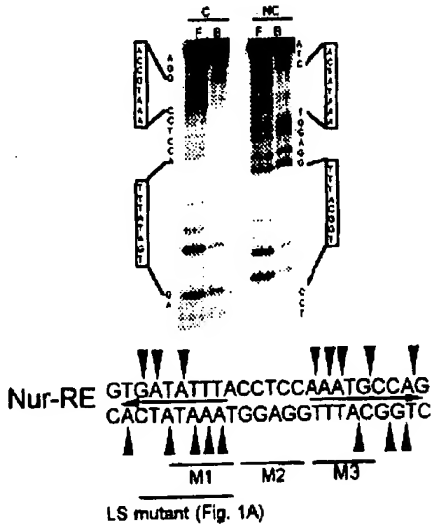

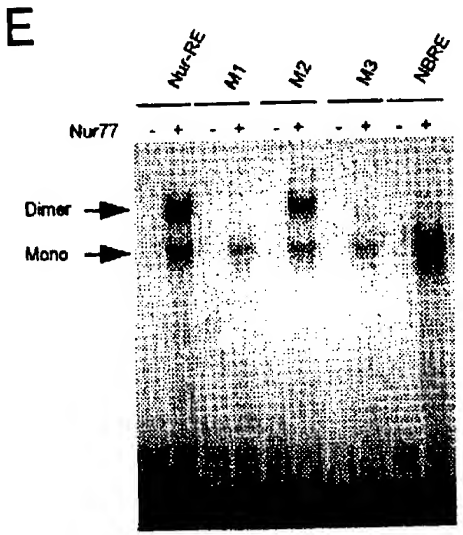

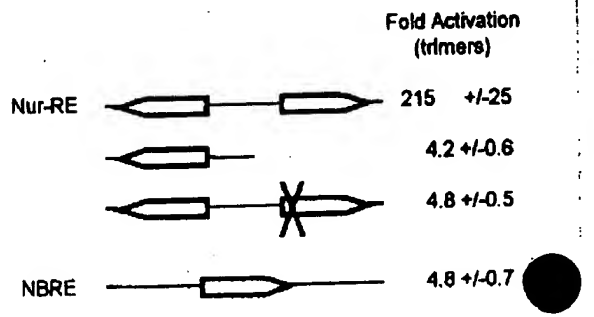

FIG. 3

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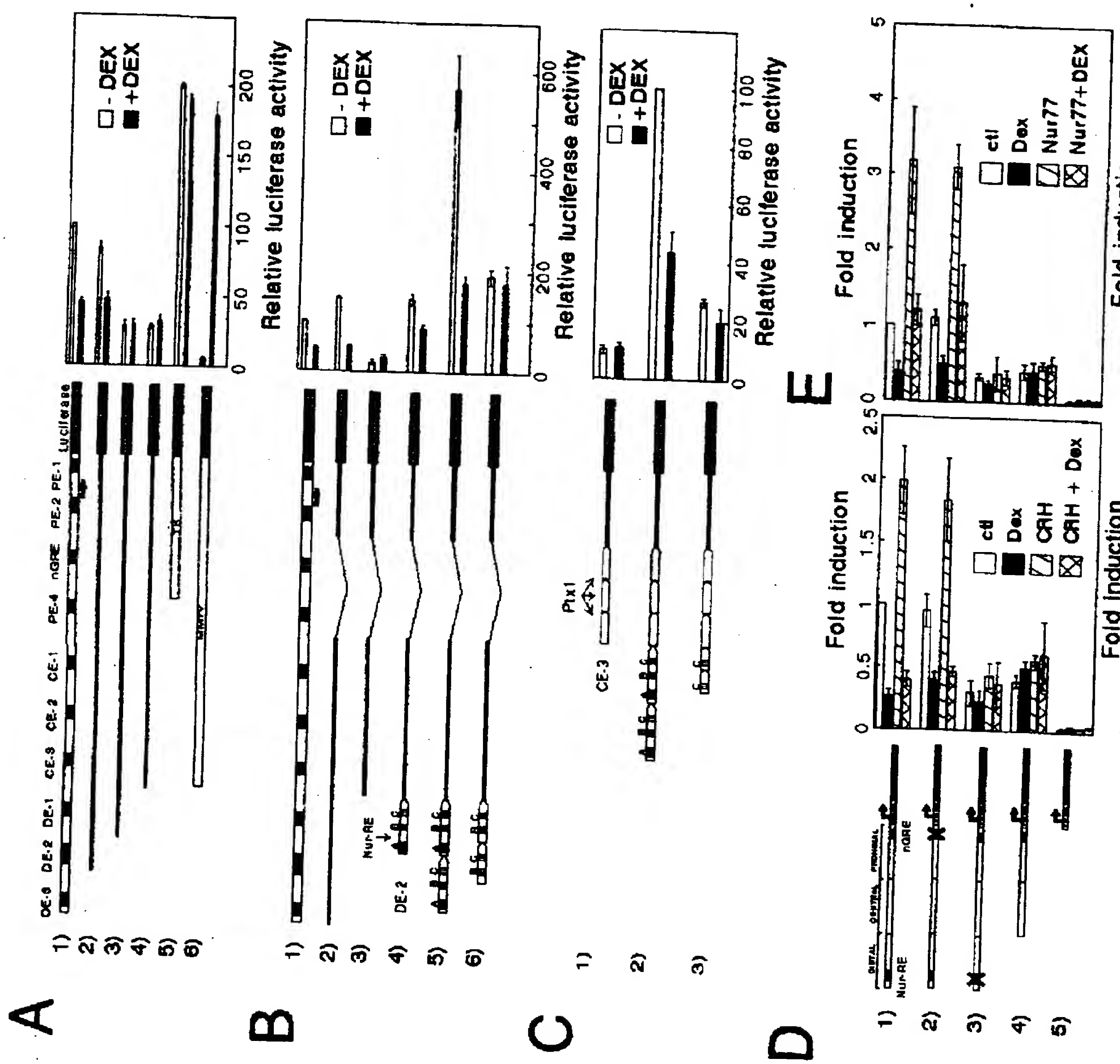
FIG. 4



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